STUDIES ON

BACILLUS PYOCYANEUS

by

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Introduction

The observation that HCN is formed in protein material undergoing decomposition has been made by numerous investigators. More recently Emerson, Bailey and Cady (1) reported the presence of HCN in eggs undergoing putrification and showed that its production could be maintained by transfering some of this material to fresh egg material. A bacterial examination of this material was made by Clawson and Young (2) who isolated and identified the etiological factor as Bacillus Pyocyaneus. They used the modified Schönbein test to detect the presence of HCN and the Prussian blue reaction for confirmation. Kendall, Day and Walker (3) included Bacillus Pyocyaneus with other bacteria in some of their studies on bacterial metabolism. Patty made a comparative study of nine strains of Bacillus Pyocyaneus. Among other things he determined the optimum reaction for the production of cyanide and com-

pared quantitatively the amounts produced by these nine strains. He found the optimum Ph value to be 5.4-5.8 and that the maximum amount of HCN was produced in gelatin. Almost as satisfactory results were obtained when he used a semi-synthetic medium. He reported that the sulphocyanate colorimetric method of estimating minute amounts of HCN is a satisfactory one, providing controls are always run, as sulphocyanate is formed in protein media upon distillation in sufficient quantities to give a positive reaction.

The purpose of the work of this paper may be summarized as follows:

1. A quantitative estimation of the HCN production of eighteen additional strains of Bacillus Pyocyaneus by the sulphocyanate method.

2. A comparison of the production of HCN by Bacillus Pyocyaneus and Bacillus Fluorescens liquefaciens both quantitatively and qualitatively.

3. To determine whether HCN is produced in a true synthetic medium and to compare the properties of growth and pigmentation with cyanide production.

Technique

The technique of the sulphocyanate method is as follows:

To a twenty-four hour agar slant growth of the organism a cubic centimeter of sterile iso-tonic salt solution was added and an emulsion made by vigorous shaking. One tenth of a cubic centimeter of this suspension was added by means of a sterile pipette to ten cubic centimeters of sterile semi-synthetic media and the same incubated twenty-four hours at 37° Centigrade. In the same manner one tenth of a cubic centimeter of this culture was added to another ten

cubic centimeter portion of semi-synthetic medium and the latter incubated twenty-four hours at 37° Centigrade. On the fifth day, after the four successive inoculations, one tenth of a cubic centimeter of the last inoculation was added to each of two 250 cubic centimeter flasks containing 100 cubic centimeters of the sterile semi-synthetic media. Four flasks connected in parallel, each fitted with two hole stoppers, were aerated with air filtered through soda lime and 4% Potassium Hydroxide solution by the suction from a filter pump. Each flask was also aerated into a solution of 4% Potassium Hydroxide to prevent the escape of any HCN.

After seven days aeration the Potassium Hydroxide solution was added to the flasks and the contents of the flask transferred to a distilling flask. The contents of the distilling flask were made acid with concentrated sulfuric acid added through a

thistle tube, and distilled over a water bath into 4% potassium hydroxide solution. To the distillate one cubic centimeter of yellow ammonium sulfide was added and the distillate evaporated to dryness on a water bath. The residue was taken up with three ten cubic centimeter portions of acetone. The acetone solution was evaporated and the residue taken up with twenty-five cubic centimeters of distilled water. To the aqueous solution fifteen drops of 5% ferric chloride was added. This solution was compared in a colorimeter with a standard potassium sulphocyanate solution to which fifteen drops of 5% ferric chloride was likewise added. The standard solution was made up so that one cubic centimeter of the standard was equal to .01 milligram of HCN acid.

Each determination was made in duplicate.

Media

The culture medium used for the first part of this work was semi-synthetic. It contained .5% peptone, .5% dextrose and .5% di-potassium hydrogen phosphate. (5)

The true synthetic medium used for the latter part of the work consisted of Tyrode solution as recommended by Sollman (6) except that only one half of the calcium was used. Tyrode solution consists of sodium chloride .8%, potassium chloride .02%, sodium bi-carbonate .1%, magnesium chloride .01%, di-sodium hydrogen phosphate .005%, glucose .1% and crystalline calcium chloride .02%. The calcium chloride must be added last in solution to prevent the formation of a precipitate.

The three modifications of this medium which I used were as follows:

MODIFICATION 1 - Tyrode plus .2% ammonium succinate.

MODIFICATION 2 - Tyrode plus .05% tyrosine.

MODIFICATION 3 - Tyrode plus .2% ammonium succinate, and .05% tyrosine.

All the flasks of the true synthetic media and its modifications were adjusted to Ph 7.

The source of the strains used in this work are given in table I.

Discussion

The quantity of HCN produced by different strains of B. Pyocyaneus varies from a trace to several hundredths of a milligram per 100 c.c. of medium as shown by table II. One or two strains produced nearly a tenth of a milligram of HCN. In the determination of the amounts of cyanide produced by strains 68 and 178,

Patty and myself do not agree. My results are about one fifth as large as those obtained by him. The remainder of my determinations and those reported by Patty compare favorably. The discrepencies in the amounts reported in these two investigations may be due to several factors. First to error in the original work done, second to differences of the medium used or third in a flucuating variability of cyanide production by the different strains in a given medium. In my opinion the latter is the more probable explanation of the differences in amounts reported for the following reasons. First, Patty's findings as well as my own were the results of repeated and consistent determinations. Second, the technique and the culture media were essentially the same. Third, the results obtained by Patty for seven out of nine strains re-

ported by him are in accord with my findings for eighteen additional strains.

From a perusal of table II it will be noted that B. Fluorescens liquefaciens produced a relative large amount of HCN, that is .05126 milligram per 100 cubic centimeters of culture media. This is almost equivalent to the maximum amount produced by several strains of B. Pyocyaneus. The production of cyanide cannot therefore be used as a means of differentiation between B. Pyocyaneus and B. Flourescens liquefaciens.

The true synthetic medium used brought out some interesting points in relation to cyanide production. Tyrode solution which is a combination of inorganic salts was not favorable to cyanide production. The addition of Tyrosine likewise did not stimulate its formation.

However, when ammonium succinate was added to tyrode solution, a small amount was produced with the addition of tyrosine to ammonium succinate and tyrode solution the amount of HCN produced was increased five fold. This, however, was only one fourth of the maximum production in the semi-synthetic medium.

It might be augued that the absence of HCN in cultures grown in tyrode solution and tyrode plus tyrosine could be accounted for by the difference in growth. From an examination of table V it will be seen that the turbidity was about one fourth of that where ammonium succinate was used alone, or in conjunction with tyrosine. Apparently, there was sufficient growth to warrant a trace of HCN, providing conditions had been favorable for its production. It is interesting to note that the combination of

ammonium succinate and tyrosine was more favorable for HCN production than when ammonium succinate was used alone. A somewhat similar parallelism existed in respect to pigmentation.

No pigment was produced in tyrode or the same plus tyrosine. A decided greenish pigment was formed when ammonium succinate was substituted for tyrosine. This was changed to a dark blue-green color, when ammonium succinate was used in conjunction with the latter.

Summary

1. Of the eighteen strains of B. Pyocyaneus studied, all produced HCN, the amount varying from a trace to a tenth of a milligram per 100 cubic centimeters of the culture medium.

2. B. Fluorescens liquefaciens produces a relative large amount of cyanide. Therefore HCN production cannot be used as a means of differentiation between B. Pyocyaneus and B. Fluorescens liquefaciens.

3. A synthetic medium composed of $NaCl$, KCl, $NaHCO_3$, $MgCl_2$, Na_2HPO_4, Dextrose, and $CaCl_2$ permitted of some growth, but was not favorable to cyanide production. The addition of ammonium succinate to this medium facilitated HCN production to a slight extent. When, however, tyrosine as well as ammonium succinate was added, the production of HCN was increased five fold. This, however, was only one fourth of the cyanide production in a semi-synthetic medium.

Table I

Source of the Strains used.

Cultures:		
250	American Museum	No. 150
251	" "	" 151
252	" "	" 152
253	" "	" 156
254	" "	" 206
255	" "	" 102
	Dawson Strain, Bureau of Animal Industry.	
256	American Museum	No. 129
	Jackson Strain	
257	Lister Institute	- Bullock Strain 42A
258	" "	- Goat Strain 254
259	" "	- Fildes Strain 150B

Table I (continued)

260	From a case of Influenza K.U.
261	" " " " Typhoid - Lawrence
262	" " " " " "
263	Oysters
264	Minnesota State Board of Health
265	Kansas " " " "
266	" " " " "
267	" " " " "
268	Sausage
68	Kansas University Stock Culture
178	Gizzard Strain.

Table II

No. of Culture		Readings Standard	Readings Unknown	Dilution of Standard	HCN in Mgms	Ave. HCN Content of Medium	Actual HCn Produced
250	1	40	21.8	1-40	.04348	.04468 : .01762Mg.	.02706
	2	40	23.0		.04587		
251	1	30	20.5	1-40	.02460	.02528 : .01762	.00766
	2	30	28.9		.02595		
252	1	40	25.6	1-40	.03906	.03803 : .01762	.02041
	2	40	27.3		.03700		
253	1	20	35.7	1-20	.02801	.02831 : .01762	.01069
	2	20	34.6		.02861		
254	1	30	37.7	1-20	.0400	.04175 : .01762	.02413
	2	30	34.5		.0435		
255	1	30	16.7	1-20	.08981	.09772 : .01762	.08010
	2	30	14.2		.10563		
256	1	40	26.6	1-40	.03760	.03672 : .01762	.01910
	2	40	27.9		.03584		
257	1	30	22.2	1-40	.03380	.03594 : .01762	.01832
	2	30	19.7		.03807		
258	1	30	25.5	1-20	.05882	.06092 : .01762	.04330
	2	30	23.8		.06302		
259	1	40	29.0	1-40	.03450	.03450 : .01762	.01688
-	2	40	28.7		.03450		
260	1	30	32.2	1-20	.04658	.04457 : .01762	.02695
	2	20	23.5		.04255		

Table II (continued)

No. of Culture		Readings Standard	Readings Unknown	Dilution of Standard	HCN in Mgms	Ave.	HCN Content of Medium	Actual HCN Produced
261	1	40	29.4	1-20	.06802	.06712	.01762	.04950
	2	40	30.2		.06622			
262	1	40	30.8	1-40	.03246	.03312	.01762	.01550
	2	40	29.6		.03378			
263	1	40	24.4	1-20	.08197	.07304	.01762	.05542
	2	30	23.2		.06410			
264	1	30	34.4	1-20	.04360	.04235	.01762	.02473
	2	30	36.5		.04109			
265	1	40	26.4	1-20	.07575	.07428	.01762	.05666
	2	30	20.6		.07281			
266	1	30	24.5	1-20	.06122	.05061	.01762	.03299
	2	30	20.6		.05000			
267	1	40	35.7	1-40	.02801	.02879	.01762	.01118
	2	40	33.8		.02958			
178	1	30	37.2	1-40	.02661	.02715	.01762	.00953
	2	30	36.1		.02770			
68	1	30	20.4	1-20	.07353	.07687	.01762	.05925
	2	30	18.7		.08021			
B. Flourecens Liq.	1	30	31.3	1-20	.07042	.06888	.01762	.05126
	2	40	29.7	1-20	.06734			
Check		40	56.4	1-40	.01773			
		40	57.1	1-40	.01762			

Table III

	Mgms HCN Produced	Total N of Medium	% HCN of Total N Available
250	.02706	30.8	.00088
251	.00766	30.8	.00025
252	.02041	"	.00060
253	.01069	"	.00035
254	.02413	"	.00078
255	.08010	"	.00260
256	.01910	"	.00062
257	.01832	"	.00059
258	.04330	"	.00140
259	.01688	"	.00054
260	.02695	"	.00087
261	.04950	"	.00160
262	.01550	"	.00050
263	.05542	"	.00180
264	.02473	"	.00080
265	.05666	"	.00187
266	.03299	"	.00107
267	.01118	"	.00036
68	.05925	"	.00192
178	.00953	"	.00031
Flourescence Liq.	.05126	"	.00166

Table IV

Culture 68	Readings Standard	Readings Unknown	Dilution of Standard	HCN in Mgms	Less Check
Tyrode	20	39.9	1-40	.01253	.00000
Tyrode + Tyros	20	40.3	1-40	.01240	.00000
Tyrode + NH_3 Succ	20	32.4	1-40	.01543	.00283
Tyrode Tyroscine NH_3 Succ	20	18.3	1-40	.02732	.01472
Tyrode Check	20	39.6	1-40	.01260	

Table V

Culture 68	Growth 48 hr	Growth 7 da	Pigmentation 48 hr	Pigmentation 7 da
Tyrode	-	Slight +	-	-
Tyrode + Tyrosine	-	Slight +	-	-
Tyrode + NH_3 Succ	4 +	4 +	4 + green	4 + green
Tyrode Tyrosine NH_3 Succ	4 +	4 +	+ blue-green	+ blue-green

BIBLIOGRAPHY

1. Emerson, Bailey and Cady. 1913.
On the Formation of Hydrocyanic Acid from Proteins. Journal of Biological Chemistry. Vol. 15. No. 3.

2. Clawson and Young, 1913.
Preliminary Report on the Production of Hydrocyanic Acid by Bacteria: Journal of Biological Chemistry. Vol. 15, No. 3.

3. Kendall, Day and Walker, 1914.
Studeis on Bacterial Metabolism: Journal of American Chemical Society. 36.

4. Patty, 1921.
The Production of Hydrocyanic Acid by Bacillus Pyocyaneus: Journal of Infectious Diseases: Vol. 29, No. 1.

5. Standard Methods of Water Analysis, 1917.